Tab 1. Biochemical profiles in normal persons *vs* patients with hepatocellular carcinoma. $\bar{x}\pm s$. $^aP>0.05$, $^bP<0.05$, $^cP<0.01$ *vs* control.

	Sodium/ mmol L^{-1}	Potassium/ mmol L^{-1}	Chloride/ mmol L^{-1}	Bicarbonate/ mmol L^{-1}	Creatinine/ mg L^{-1}	Uric acid/ mg L^{-1}	Urea/ mg L^{-1}	Urea-N mg L^{-1}	Residual N mg L^{-1}
Control $n=25$	137.5±1.6	4.0±0.4	98.3±2.2	25.4±1.1	8.7±1.6	42±6	282±69	13±3	24±3
Patients $n=50$	106±16[c]	4.5±0.7[c]	95±4[c]	28.5±1.1[c]	20±6[c]	82±17[c]	135±25[c]	6.3±1.6[c]	16.8±1.8[c]
Patients $n=25$	114±18[c]	4.2±0.7[c]	96±5[c]	27.4±1.2[c]	14±5[c]	68±11[b]	130±20[c]	6.1±0.9[c]	16.5±1.0[c]

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Relationship between platelet density and platelet aggregation, ATP release, and cytosolic-free calcium mobilization in rabbits

LI Bai-Yan, LI Wen-Han
(*Department of Pharmacology, Harbin Medical University, Harbin 150086, China*)
BAI Ying (*Department of Neurology, The First City Hospital of Harbin, Harbin 150010, China*)

AIM: To study the relationship of platelet density and platelet functions, including aggregation, ATP release, and intracellular calcium mobilization. **METHODS**: Platelet density was evaluated by discontinuous gradients of Percoll and cytosolic-free calcium concentration in single platelets was measured by fluorescence and imaging. **RESULTS**: Rabbit platelets were assorted into 3 subpopulations using discontinuous gradients of Percoll: high density (HD, >1.062, 20 %—30 %), intermediate density (ID, around 1.057, 40 %—50 %) and, low density (LD, <1.051, 10 %—15 %). The sizes of platelets in these subpopulations were widely different (HD: 725±76; ID: 443±47; and LD: 307±46) and well correlated with the density ($r=0.988$, $P<0.01$). Thrombin 0.5 U-induced aggregations in the 3 subpopulations were 78±8 %, 69±4 %, and 62±3 %, respectively and a

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difference was found between HD and LD ($P < 0.05$). The amounts of ATP released during aggregation were 4.7 ± 0.9, 3.4 ± 1.0, and 2.6 ± 0.6 μmol/4 10^5 platelets in HD, ID, and LD groups, respectively. Thrombin 150 U-induced $[Ca^{2+}]_i$ mobilization in HD (990 ± 130 nmol L^{-1}) was different ($P < 0.01$) from those in ID (410 ± 80 nmol L^{-1}) and LD (400 ± 40 nmol L^{-1}). Serotonin (5-HT) 3 μmol L^{-1} also caused $[Ca^{2+}]_i$ mobilization in single-washed platelet subpopulations and results were similar to those of thrombin although it did not induce any detectable aggregation or release reaction.

CONCLUSION: Platelet functions and intracellular calcium concentraotion were increased with platelet density in rabbits.

KEY WORDS blood platelets; platelet aggregation; adenosine triphosphate; calcium; thrombin; serotonin

Platelets are heterogeneous with respect to density, size, age, volume, ultrastructure, biochemical composition and function. But the interpretation of platelet density heterogeneity remains controversial. The platelet density was determined primarily by its concentration of platelet specific granules[1], including dense granules and α-granules[2, 3]. Some investigators showed that platelet density decreased during the platelet lifespan[4, 5]. However, recent studies suggested that platelet aging was accompanied by a slight increase in platelet density[6, 7], varying with the species and methods used for separating the platelet density subpopulations. The cytosolic-free calcium plays a crucial role in the processes of the signal-transducing system of platelets, the rearrangement of the microtubule and microfilament systems upon the platelet morphological change with formation of pseudopods and binding of fibrinogen to its receptors, GP $\mathrm{II_b/III_a}$, during aggregation[8]. In the present experiments, the relationships were investigated in platelet density, size, and the responses to thrombin and 5-HT, including aggregation, release reaction, and cytosolic-free calcium mobilization in washed rabbit platelets by using discontinuous Percoll gradients centrifugation.

MATERIALS AND METHODS

Agents A series of concentrations of Percoll (Pharmacia Co, Tokyo) solution was prepared for gradients centrifugation. Luciffern-Lucifease (Chrono-Log Co, Havertown PA) for measuring the secretion of ATP was dissolved in saline (40 g L^{-1}). ATP (Sigma Co, St Louis) for the typical change of platelet release reaction was dissolved in distilled water (1 mmol L^{-1}) and diluted to 300 μmol L^{-1} with saline (final concentration: 1 μmol L^{-1}) before use. Thrombin (Topical, Bovine Origin, Parke-Davis Division of Warner-Lambert Co, Morris Plains NJ) was kept at −20 C and dissolved in HEPES-buffer before use. 5-HT (Wako Pure Chemicals, Osaka) dissolved in HEPES-buffer. Egtazic acid (Sigma Co, St Louis) was dissolved in distilled water with NaOH 0.5 mol L^{-1}, pH adjusted to 7.4 with HCl 0.5 mol L^{-1}, Fura-2 and Fura 2-AM (Wako Pure Chemicals, Osaka) were used for Ca^{2+} calibration curve and platelet loading.

Preparation of platelet subpopulations Blood (60 mL in 2 plastic tubes) was collected from the carotid artery of New Zealand white rabbits (2.8 ± 0.4 kg) with 1/7 volume of acid citrate-dextrose solution (ACD; sodium citrate 85 mmol L^{-1}, citrate acid 71 mmol L^{-1}, and glocose 110 mmol L^{-1}) under ether anesthesia and then centrifuged at 200 · g at 20 — 25 C for 15 min to get platelet-rich plasma (PRP) which was further centrifuged at 800 · g for 20 min. The platelet pellet was suspended with HEPES buffer (NaCl 145, KCl 5, $MgSO_4$ 1, glucose 5 and HEPES 10 mmol L^{-1}, pH 7.4 at 22 C). The suspension was layered in a density tube containing different concentrations (60 %, 50 %, 45 %, 40 %, and 20 %) of Percoll solution. The tube was centrifuged at 210 · g at 25 C for 30 min to separate the subpopulations of platelets. Three subpopulations of platelets of high

density (HD) $\geq$ 1.062, intermediate density (ID) 1.057, and low density (LD) $\leq$ 1.051 were transferred gently into 3 separate tubes and washed again with 10 mL of HEPES buffer and centrifuged again to remove the Percoll solution. The subpopulations pellets were resuspended in HEPES buffer to a final concentration of 6×10^{11} platelets L^{-1}.

Platelet aggregation The platelet aggregation was quantified by the light transmission method[9] in 4-channels aggregometer (PAT-4A, Nippon Denshi Kagaku Co, Tokyo) and recorder (T-626DS, Nippon Denshi Kagaku Co, Tokyo). The procedures for determination of platelet aggregation were as follows: After 2 min of preincubation at 37 C, the washed platelets (250 μL) in the cuvette was further incubated for 2 min with HEPES-buffer or agents (20 μL) prior to the addition of aggregation agents (10 μL) with stirring by a siliconized magnetic bar.

Determination of ATP The ATP release in the medium from the dense granules during platelet aggregation of platelets was assessed by luminescence[10] in a 2-channels lumi-aggregometer (Chrono-Log Corp, Havertown PA) and recorder (B-281L, Rikadenki Kogyo Co, Tokyo). The amount of ATP released was expressed as μmol/4 $\times10^5$ platelets.

Fura-2 loading The washed platelets were prepared in final concentrations of 1.2×10^{12} platelets L^{-1}, 6×10^{11} platelets L^{-1} (2-fold dilution), and 3×10^{11} platelets L^{-1} (4-fold dilution) and loaded with Fura 2-AM 2 μmol L^{-1} (final concentration) at 37 C for 15 min.

In the following experiments, 3 subpopulation of washed platelets were diluted into $6\cdot10^{11}$ platelets L^{-1} and loaded with Fura 2-AM 2 μmol L^{-1} at 37 C for 15 min for this concentration of platelets showed higher $[Ca^{2+}]_i$ mobilization with lower resting level of $[Ca^{2+}]_i$. The Fura-2 loaded platelet pellet was obtained by centrifugating the platelet suspensions at 800 $\times g$ for 20 min at 25 C and resuspended in Ca^{2+}-free HEPES buffer to obtain the final concentration of $2\cdot10^{11}$ platelets L^{-1}. Extracellular Ca^{2+} concentration was adjusted to a final concentration of 1 mmol L^{-1} at 5—10 min before measuring $[Ca^{2+}]_i$ mobilization using a digital image fluorescence microscope, a computer assistant imaging and analyzing system.

Determination of platelet size The video images were obtained using a silicon-intensified target camers, with the output digitized to a resolution of 512×480 pixels by an image video analyzer with each point in the image being assigned a value from 0 to 255 in both X and Y axis depending on fluorescent intensity.

Measurement of $[Ca^{2+}]_i$ in single platelet The Fura-2 loaded washed platelet suspension (50 μL) was layered on a thin micro-cover glass (40 · 50 mm, thickness: 0.17 — 0.25 mm, Matsunami Glass Co, Tokyo) in a 2.5 mm cyclic chamber in diameter at room temperature and scartinized under a digital image fluorescence microscope (IMT2-OSP-II, Olympus®, Tokyo). The fluorescence of Fura-2 loaded single washed platelet, due to excitation at either 340 nm or 380 nm, was imaged using UV. Apr 100 · and inverted into microscope, the video images were obtained using a silicon-intensified target camera (SIT, Olympus®) which were stored and digitally analyzed by photonic microscope system (Argus 100/AC, Hamamatsu Co, Tokyo). The ratio (R340/380) image was achieved through the division of 340 nm image by 380 nm image. To evaluate how long the preparation of washed platelets could be used during experiment, the samples were made one by one at a time interval of 30 min and the results of resting level of intracellular calcium and $[Ca^{2+}]_i$ mobilization induced by thrombin 150 U were observed.

Statistical analysis All data were calculated by Statview SE + Graphics software and Excel 3.0 running in Apple Macintosh LC III personal computer, and expressed as $\bar{x}\pm s$. The differences were evaluated by t test.

RESULTS

Fluorescent difference Fluorescent ratios were different in preparations with different unmber of washed platelets loaded with Fura 2-AM 2 μmol L^{-1}. The result showed that 2-fold diluted preparation had a lower resting ratio and the highest ratio value after exposed to thrombin 150 U (Fig 1A), and those were maintained about 3 h after getting preparations (Fig 1B).

Size and density of subpopulations HD, ID, and LD platelets were fixed at the border between 60 % and 50 % (1.062) of Percoll

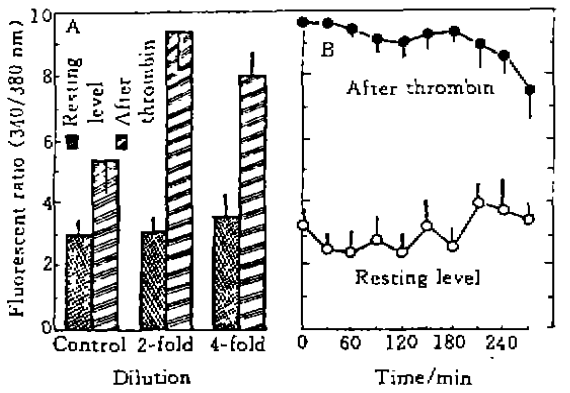

Fig 1. Effect of platelet counts on fluorescence intensity (A) and effect of sample standing time on fluorescence intensity (B). Thrombin 150 U. $n=11-15$, $\bar{x}\pm s$.

solution, 45 % (1.057) of Percoll solution and the border between 30 % (1.051) and 20 % of Percoll solution respectively in the density tube after centrifugation, and the size of platelets were measured by X and Y axis and expressed by the area ($X\times Y$) in all subpopulations (Tab 1). The results showed that platelet size was well correlated with that of its density (Fig 2B).

Tab 1. Size in density subpopulations of washed platelets. $n=54-73$ cells in each subpopulation from at least 6 rabbits, $\bar{x}\pm s$.
$^bP<0.05$ *vs* LD and $^fP<0.01$ *vs* LD and ID.

Density	Size	Density	% in all population
High	725 ± 76^b	>1.062	20—30
Intermediate	443 ± 47^f	1.057	40—50
Low	307 ± 46	<1.051	10—15

Platelet aggregation In thrombin 0.5 U-induced platelet aggregation, the change of light transmission was the most remarkable in HD subpopulation (Tab 2).

ATP release Thrombin-induced platelet aggregation was accompanied by secretion of ATP from the dense granules of platelets (Tab 2).

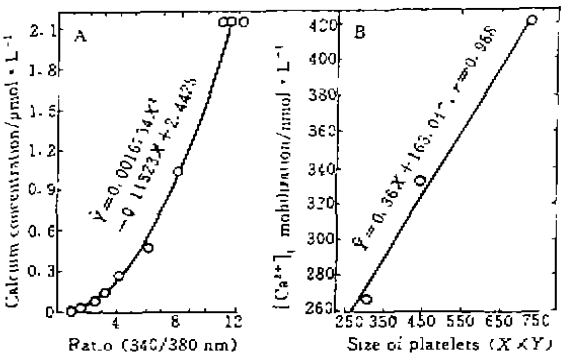

Fig 2. Standard calibration curve of calcium concentration (A) and the relationship between size of platelets and thrombin 15 U-induced $[Ca^{2+}]_i$ mobilization (B).

Tab 2. Thrombin 0.5 U-induced aggregation and release reaction in density subpopulations of washed platelets. $n=12-14$ samples in each subpopulation from at least 6 rabbits, $\bar{x}\pm s$. $^bP<0.05$ *vs* LD. $^fP<0.01$ *vs* LD and ID. $^iP<0.01$ *vs* LD.

Deosity	Aggregatioo/% (Light transmission)	ATP release, ATP μmol/4×10^5 platelets
HD	78 ± 8^i	4.7 ± 0.9^f
ID	69 ± 4^b	3.4 ± 1.0^f
LD	62 ± 3	2.6 ± 0.6

$[Ca^{2+}]_i$ mobilization by thrombin $[Ca^{2+}]_i$ in single washed platelets was calculated from R340/380 using standard calibration curve of calcium with Fura-2 2 μmol L^{-1} (Fig 2A).

The intracellular calcium did not show a significant difference in 3 subpopulations of platelets about 80 nmol L^{-1} (Fig 3A), but the $[Ca^{2+}]_i$ mobilizations by thrombin were not the same. $[Ca^{2+}]_i$ mobilizations in HD platelets were very sensitive to thrombin 15 and 150 U and concentration-dependent, while in ID and LD groups, $[Ca^{2+}]_i$ mobilizations by the same concentration of thrombin were only about 400 nmol L^{-1}, less than the half value in HD.

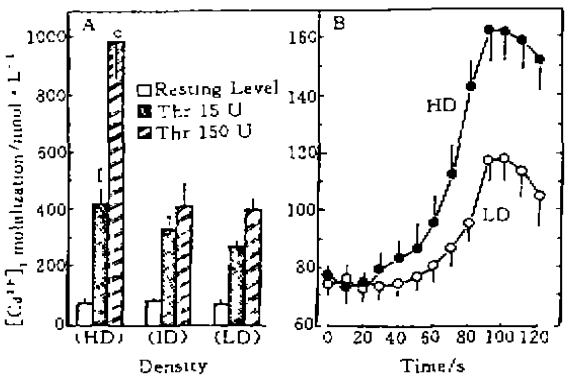

Fig 3. (A) $[Ca^{2+}]_i$ **mobilizations induced by thrombin (Thr, $n=15-21$) and (B) $[Ca^{2+}]_i$ mobilization by 5-HT 3 μmol L^{-1}.**
$n=26-31$ in HD and LD subpopulations. $\bar{x}\pm s$.
$^cP<0.01$ *vs* LD or ID, $^tP<0.01$ *vs* LD.

$[Ca^{2+}]_i$ mobilization by 5-HT The addition of 5-HT to the samples resulted in a slight $[Ca^{2+}]_i$ mobilization in single washed platelet. In HD platelets, $[Ca^{2+}]_i$ concentration was elevated from basal level (76 ± 6 nmol L^{-1}) to peak value (162 ± 15 nmol L^{-1}) at 90 min after the administration of 5-HT 3 μmol L^{-1} (Fig 3B).

DISCUSSION

In this work we demonstrated that the fluorescence intensity of Fura 2-AM loaded washed platelets were closely associated with the platelet counts when the concentration of Fura 2-AM was fixed. In other words, to exactly show the $[Ca^{+}]_i$ level, the concentration of Fura 2-AM must be high enough for loading the cytosolic-free calcium, but it is still unclear if high concentration of Fura 2-AM have a cytotoxity, at least, high concentration of Fura 2-AM reduced the platelet aggregation due to chelating effect of Fura 2-AM[11], suggesting that both platelet counts and concentration of Fura-2/AM were very important for getting a good fluorescence intensity.

In addition, the fluorescence intensity of both basal level or the level after stimulation by thrombin increased or decreased with the prolongation of sample standing time after Fura 2-AM loading, indicating that washed platelets were exhausted gradually and the function was getting down time-dependently. So, in this experiment, the measurement of intracellular calcium concentration were performed within 3 h after the preparation of washed platelets.

The results also demonstrated that the size of the platelet increased with its density. This data was supported by a previous report [7], and primarily as a result of differences in the number of storage organelles revealed by a significant decrease in dense body number and endogenous serotonin content with decreasing platelet densities[1].

The present study showed that the responses of both platelet aggregation and release reaction to thrombin measured simultaneously in HD platelets were functionally stronger and more active than those of in LD platelets. This result was consistent with a previous report[12]. The significant reduction in platelet function with decrease in density may be due to partial or complete degranulation during platelet lifespan[13]. Further experiment showed that intracellular calcium mobilization induced by thrombin was significantly much higher in HD than those in ID and LD without change of the basal level, a similar result was also found by using exogenous serotonin.

In conclusion, the heterogenicity of platelet is closely related to its size, density and function. the most dense platelets are much larger in size and more active in function. In general, the density decreased gradually as the platelets increased in age in circulation since the platelets were exposured to

the stimulants repeatedly, resulting in decrease in size and reduction in function. From this opinion, the results of this study supported the view that the highest density platelets were newly formed ones with more active function[2,3]. This study also suggested that why the number of larger platelets increased in some pathologic states with atherosclerosis cardiovascular disease, such as myocadial infarction[14], while decreased in hemorrhagic disorders, such as Wiskott-Aldrich syndrome[15].

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家兔血小板密度与其聚集和 ATP 释放反应及细胞内游离钙动员的关系

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李柏岩，李文汉（哈尔滨医科大学药理教研室，哈尔滨 150086，中国）

白 颖（哈尔滨市第一医院神经内科，哈尔滨 150010，中国）

目的：研究血小板密度与其功能及单细胞内游离钙动员的关系. **方法**：以 Percoll 间断梯度离心法及萤光图像法评价和测量血小板密度及细胞内钙浓度. **结果**：家兔血小板可分为高(HD)，中(ID)和低(LD)三个密度亚群，各亚群之间大小明显差异，且与密度相关($r=0.988$，$P<0.01$)。0.5 U 凝血酶的聚集和 ATP 释放反应 HD 均较 LD 强. 150 U 凝血酶刺激后，单血小板内游离钙水平 HD 明显高于其它亚群($P<0.01$)。尽管血清素 3 μmol L^{-1}无聚集和释放反应，但对细胞内游离钙影响的结果与凝血酶相似。**结论**：家兔血小板的功能及单细胞内游离钙水平随血小板的密度增加而增强.

关键词 血小板；血小板聚集；腺苷三磷酸；钙；凝血酶；血清素